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Modification of metal bioaccumulation and toxicity in *Daphnia magna* by titanium dioxide nanoparticles

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ABSTRACT

Titanium dioxide (TiO_2) nanoparticles are widely used in water treatments, yet their influences on other contaminants in the water are not well studied. In this study, the aqueous uptake, assimilation efficiency, and toxicity of two ionic metals (cadmium-Cd, and zinc-Zn) in a freshwater zooplankton, *Daphnia magna*, were investigated following 2 days pre-exposure to nano-TiO_2. Pre-exposure to 1 mg/L nano-TiO_2 resulted in a significant increase in Cd and Zn uptake from the dissolved phase. After the nano-TiO_2 in the guts were cleared, the uptake rates immediately recovered to the normal levels. Concurrent measurements of reactive oxygen species (ROS) and metallothioneins (MTs) suggested that the increased metal uptake was mainly due to the increased number of binding sites provided by nano-TiO_2 presented in the guts. Consistently, pre-exposure to nano-TiO_2 increased the toxicity of aqueous Cd and Zn due to enhanced uptake. Our study provides the evidence that nano-TiO_2 in the guts of animals could increase the uptake and toxicity of other contaminants.

1. Introduction

Titanium dioxide (TiO_2) nanoparticles are found in many of today's industrial, commercial and consumer products, including self-cleaning surface coatings, light-emitting diodes, solar cells, disinfectant sprays, sporting goods, water treatment agents, and topical sunscreens (EPA, 2009). It is estimated that worldwide production of nano-TiO_2 will reach 2.5 million tons by 2025 (Robichaud et al., 2009). Compared to the conventional TiO_2, nano-TiO_2 have a larger surface area-to-volume ratio and offers higher adsorption and photo-catalytic oxidation efficiencies. Thus, nano-TiO_2 may be used in the removal of arsenic (by converting arsenite [As(III)] to arsenate [As(V)]) (Li et al., 2009), disinfection of pathogens (Alrousan et al., 2009), and degradation of organic compounds (Han et al., 2009). Although many current foods (e.g., sweets and chewing gums) and personal care products (toothpastes and sunscreens) do not carry the 'nano' label, most of them certainly contain TiO_2 nanoparticles (Westerhoff et al., 2012). With their widespread applications and usages, nano-TiO_2 will inevitably find its way to the aquatic systems. Thus it is essential to examine the harm they could potentially inflict on aquatic organisms.

Considerable efforts have been made to investigate the potential adverse effects of nano-TiO_2 on various organisms in the water [such as algae (Aruoja et al., 2009; Hartmann et al., 2010), water flea (Lovern and Klaper, 2006; Lovern et al., 2007), and fish (Federici et al., 2007; Johnston et al., 2010)]. Generation of reactive oxygen species (ROS) and their inflammatory effects are considered as the main mechanisms for nano-TiO_2 toxicity (Federici et al., 2007; Long et al., 2006; Ma et al., 2012). Through adsorption, nano-TiO_2 could also interact with other contaminants and modify their behavior in the environment and organisms (Nagaveni et al., 2004), potentially causing further undesirable effects. Previously, we have demonstrated that nano-TiO_2 can act as carriers for metals such as cadmium (Cd) and zinc (Zn). We found that metals bound with nano-TiO_2 were more bioavailable to *Daphnia magna* than metals that were not due to the direct ingestion of nanoparticles and metal complexes by the daphnids (Tan et al., 2012). Another study has shown that nano-TiO_2 can significantly accumulate in the digestive system of *D. magna* (Hartmann et al., 2012) to directly interact with the ingested food and affect the assimilation efficiency (AE). Daphnids are known to 'drink' their surrounding water to facilitate digestion (Fox, 1952). This may serve as an important route for dissolved metal uptake in the digestive system. Stobbart et al. (1977) estimated that *D. magna* may replace an amount of fluid equivalent to 3.1% of their body weight every 10 min through drinking. Thus, in addition to acting as carriers, nanoparticles accumulated in the guts of daphnids may provide potential binding

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or adsorption sites for any incoming/ingested metals. However, information on the influence of nanoparticle pre-exposure on metal biokinetics and toxicity in aquatic organisms is limited.

The aim of this study was therefore to quantify the uptake, dietary assimilation, and toxicity of ionic Cd and Zn in an ecologically important freshwater zooplankton, *D. magna*, after exposure to TiO_2 nanoparticles. ROS generation and metallothionein (MT) induction as two important biomarkers were also measured. Again, we chose the two metals, Cd and Zn, mainly due to their environmental importance as metal contaminants, and because the biokinetics of these two metals in *D. magna* is already well established (Yu and Wang, 2002a, b). The results, combined with those from a previous study (Tan et al., 2012), can provide a more complete understanding of how nano-TiO_2 affect the Cd and Zn biokinetics and toxicity in *D. magna*.

2. Materials and methods

2.1. Organisms, water, and radioisotopes

D. magna were cultured in glass-fiber-filtered (GF/C Whatman, Maidstone, UK) unpolluted pond water (collected from site located at N 22°20′11.3″, E 114°15′59.4″, with pH = 8.2, Cd = 0.016 μg/L, Zn = 1.6 μg/L) (Guan and Wang, 2004a). The animals (one individual/10 mL medium) were maintained at a temperature of 23.5 °C with a light to dark cycle of 14:10 h, and fed the green algae *Chlamydomonas reinhardtii* daily at a density of 10^5 cells/mL. An artificial WC medium (containing 0.25 mM $CaCl_2$, 0.15 mM $MgSO_4$, 0.15 mM $NaHCO_3$, 0.05 mM K_2HPO_4, 1 mM $NaNO_3$, 0.1 mM H_3BO_3, trace metals and vitamin) (Guillard and Lorenzen, 1972) was used to grow the algae. At the exponential growth stage, algae were collected by centrifugation to remove the growth medium, and stored in filtered pond water at 4 °C. In all experiments, the simplified Elendt M7 medium (SM7, containing only $CaCl_2$, $MgSO_4$, K_2HPO_4, KH_2PO_4, $NaNO_3$, $NaHCO_3$, Na_2SiO_3, H_3BO_3, and KCl and without disodium ethylenediaminetetraacetic acid, trace metals, or vitamins) (Samel et al., 1999) was used. The pH of the SM7 medium was adjusted to 8.2 by adding 0.1 N NaOH to it before all experiments.

The biokinetics of the two metals was traced using the gamma radiotracers ^{109}Cd (in 0.1 N HCl, from New England Nuclear, Boston) and ^{65}Zn (in 0.1 N HCl, from Riso National Laboratory, Denmark). The radioactivity was determined with a Wallac 1480 NaI (T1) gamma detector (Turku, Finland) at 88 keV for ^{109}Cd and at 1115 keV for ^{65}Zn. All analyses were related to appropriate standards and were calibrated for spillover and radioisotope decay. The counting time was 3 min which yielded propagated counting errors of less than 5%.

2.2. Characterization of nano-TiO_2

Nano-TiO_2 powder (product number: 637254, <25 nm in particle size, 99.7% trace metals basis) was purchased from Sigma–Aldrich Corporation. According to the manufacturer, the crystal phase of nano-TiO_2 was anatase, and the specific surface area was 45–55 m^2/g with a density of 3.9 g/mL. The particle morphology and elemental composition were analyzed using transmission electron microscopy (TEM, JEOL 2010F) with energy-dispersive X-ray spectrometry (EDX) capability. The TEM equipment was operated at an acceleration voltage of 100 kV. The TiO_2 nanoparticles were dispersed in Milli-Q ultrapure water (Barnstead, Dubuque, IA, USA), and was then subjected to sonication for 20 min (50 W/liter at 40 kHz), dripped onto a cleaned 200 mesh Cu carbon grid and dried at room temperature for one day before the TEM and EDX analysis. The average diameter and the zeta potential of nano-TiO_2 (0.1 and 1 mg/L) in SM7 were determined by dynamic light scattering (DLS) with a zeta potential analyzer (ZetaPALS, Brookhaven Instruments).

2.3. Exposure to nano-TiO_2

In this study, all experiments were carried out after the daphnids were pre-exposed to nano-TiO_2 suspension for 2 days. For each experiment, there were three replicates. A stock solution of 1 g/L nano-TiO_2 was first prepared by dispersing the nanoparticles in Milli-Q ultrapure water (Barnstead, Dubuque, IA, U.S.), followed by sonication for 20 min (50 W/liter at 40 kHz). Test solutions of two different nano-TiO_2 concentrations (0.1 and 1 mg/L) were prepared in beakers (high-density polyethylene) by diluting the nano-TiO_2 stock with the SM7 medium. Then ten 14-day-old daphnids were exposed in 100 mL of the nano-TiO_2 suspension (0 as control, 0.1 and 1 mg/L) for 2 days at a temperature of 23.5 °C with a light to dark cycle of 14:10 h. At the end of exposure, daphnids were moved to a clean SM7 medium for 2 h before other experiments. Meanwhile, after 2 d exposure the accumulated nano-TiO_2 concentrations in daphnids were measured by digesting the animals with ammonium persulfate. Detailed procedures for the measurement of TiO_2 concentration are available from Khosravi et al. (2012). To quantify the dry weight of daphnids, the animals were collected at the end of the experiment and subsequently dried at 80 °C overnight.

2.4. Uptake of dissolved Cd and Zn

The uptake kinetics of Cd and Zn was quantified in the SM7 (pH = 7) medium using radiotracer techniques to ensure that the uptake of Zn and Cd was accurately measured at low exposure concentrations. Daphnids were exposed simultaneously to Cd and Zn. Two uptake experiments were conducted in this study. The first experiment investigated the dissolved uptake of Cd and Zn after the daphnids were exposed to nano-TiO_2 suspension for 2 days. Since the dissolved uptake of Cd and Zn increased significantly after the daphnids were exposed (see below), the second experiment was necessary to determine the dissolved uptake of Cd and Zn with or without a high density of algae (2×10^5 cells/mL). The first experiment used three total dissolved Cd and Zn concentrations (2.5, 10 and 20 μg/L) with three replicates. The test solutions were prepared by adding an appropriate volume of a certified $CdCl_2$ and $ZnCl_2$ stock solution (1000 μg/mL, PerkinElmer) and radiotracers (2 μCi/L for ^{109}Cd and 3 μCi/L for ^{65}Zn) to the SM7 medium. Ten 16-day-old daphnids previously exposed to nano-TiO_2 for 2 days were added to each beaker with 100 mL of test solution. No food was provided during the 8-h uptake period. At 2, 4, 6, and 8 h, the daphnids were collected onto a mesh using a wide-mouth plastic pipette, and rinsed with nonradioactive SM7 containing 1 μM Na_2EDTA for approximately 1 min to remove the spiked medium and the weakly adsorbed metals from their carapace (Yu and Wang, 2002a). Their radioactivity was measured, after which the animals were immediately returned to the exposure medium. After 8 h of exposure, the animals (10 individuals) from each treatment were collected and dried at 80 °C overnight and their dry weight were determined. The average radioactivity of the exposure medium was monitored by taking a 1 mL sample from each replicate at the beginning and end of exposure, which was used to calculate the dry-weight concentration factor (DCF, L/g dry wt., as the radioactivity in daphnids divided by the radioactivity in the medium). In the second experiment, daphnids previously exposed to nano-TiO_2 for 2 days (1 mg/L) were transferred to 100 mL of the SM7 medium with or without 2×10^5 cells/mL algae for 4 h. After that, the uptake of Cd and Zn was quantified by exposing the daphnids to dissolved metals, using the same experimental conditions as those for the first experiment.

2.5. Metallothionein (MT) and reactive oxygen species (ROS)

In order to investigate whether nano-TiO_2 induced MT and ROS, 14-day-old daphnids were first exposed to nano-TiO_2 at various concentrations (0, 0.05, 0.1, 0.2, 0.5 and 1 mg/L) for two days. There were three replicates (10 daphnids per replicate) for each treatment. At the end of the exposure, daphnids were moved to a clean SM7 medium for 2 h before the determination of MT and ROS. The MT concentration was measured using a modified silver saturation assay described by Scheuhammer and Cherian (1991), and the level of ROS generation was measured using a 2, 7-dichlorofluorescein diacetate (H_2DCFDA) dye. Detailed procedures for the measurement of MT concentration and ROS are available from Guan and Wang (2004b) and Kim et al. (2009), respectively. Whether the presence of TiO_2 NPs in daphnids could influence the accuracy of these bioassays was however not specifically tested.

2.6. Dietary assimilation of Cd and Zn

The AEs of Cd and Zn from algae (*C. reinhardtii*) in *D. magna* were quantified at three food concentrations ranging from 2.5×10^4 to 10^5 cells/mL, using ^{109}Cd and ^{65}Zn as radiotracers. The algae used in the pulse-feeding were first radiolabeled by radioactive ^{109}Cd and ^{65}Zn. Specifically, algae at the exponential phase were collected by centrifugation at 3000 *g* and resuspended in the modified WC medium (without the addition of Cu, Zn, and EDTA) spiked with ^{109}Cd (20 μCi/L) and ^{65}Zn (30 μCi/L) at an initial cell density of 5×10^4 cells/mL. After 5 days of growth, the cells were again centrifuged at 3000 *g* and resuspended in the SM7 medium. This process was repeated twice to remove the weakly bound ^{109}Cd and ^{65}Zn. After the cell density measurement using a hemocytometer, the algae were immediately used in the AE experiments. There were three replicates in each treatment, and each replicate contained 100 mL of the SM7 medium. After 20 min of feeding and radioactivity measurement, the daphnids were returned to the SM7 medium under the same algal density without radioisotopes. The radioactivity remaining in the daphnids was measured over a 36-h depuration period at intervals of 3–12 h. Water and food were renewed after each radioactivity measurement.

2.7. Time-to-death test

In order to test the influence of nano-TiO_2 exposure on Cd and Zn toxicity in *D. magna*, a time-to-death experiment was performed using an approach described by Levinton et al. (2003) and Tsui and Wang (2005). Daphnids were first exposed to nano-TiO_2 at 0.1 and 1 mg/L for 2 days. Thirty individuals were then divided into three replicates containing 100 ml of the SM7 medium with Cd or Zn. The exposed nominal Cd and Zn concentrations were 150 μg/L and 1.5 mg/L respectively. The survival of daphnids was monitored every 1 h within a 24-h period.

2.8. Statistics

Statistical analyses were performed using SPSS 16.0. All data were expressed by mean ± standard deviation. The AE values of Cd and Zn in daphnids in various treatments were compared using two-way ANOVA with 'nano-TiO_2 concentration'